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Differential incorporation of uracil DNA glycosylase UNG2 into HIV-1, HIV-2, and SIV_{MAC} viral particles

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Abstract

We have previously reported that the host uracil DNA glycosylase UNG2 enzyme is incorporated into HIV-1 virions via a specific association with the viral integrase (IN) domain of Gag-Pol precursor. In this study, we investigated whether UNG2 was packaged into two phylogenetically closely related primate lentiviruses, $HIV\text{-}2_{ROD}$ and SIV_{MAC239}. We demonstrated by GST-pull-down and coprecipitation assays that INs from HIV-1, $HIV\text{-}2_{ROD}$, and SIV_{MAC239} associated with UNG2, although the interaction of UNG2 with $HIV\text{-}2_{ROD}$ IN and SIV_{MAC239} IN was less strong than with HIV-1 IN. We then showed by Western blotting that highly purified HIV-2 and SIV_{MAC} viral particles did not incorporate host UNG2, contrasting with the presence of UNG2 in HIV-1 viral particles. Finally, we showed that HIV-1/SIV chimeric viruses in which residues 6 to 202 of HIV-1 IN were replaced by the SIV counterpart were impaired for packaging of UNG2, indicating that the incorporation of host UNG2 into viral particles is the hallmark of the HIV-1 strain. Moreover, we found that HIV-1/SIV IN chimeric viruses were deficient for viral propagation.
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Keywords: Uracil DNA glycosylase UNG2; Integrase; HIV-1; HIV-2; SIV; HIV-1/SIV IN chimeric viruses

Introduction

Uracil DNA glycosylases belongs to a family of DNA repair enzymes involved in the base excision repair (BER) pathway by specifically removing uracil residues from DNA (for a review, see Krokan et al., 1997). Human cells have been reported to contain at least six distinct enzymatic species able to efficiently excise uracil bases from DNA (Muller and Caradonna, 1993; Neddermann and Jiricny, 1994; Nilsen et al., 1997; Hendrich et al., 1999; Haushalter et al., 1999). These enzymes include mitochondrial and nuclear DNA glycosylase isoforms (UNG1 and UNG2, respectively); a cyclin A-like protein (UDG2); thymidine-DNA glycosylase (TDG); MBD4, a mismatch-specific U/T:G-DNA glycosylase; and SMUG1, a glycosylase having a preference for uracil present in single-stranded DNA substrates. In addition to these enzymatic activities, another distinct class of protein coexists in cells, the deoxyuridine triphosphatase (dUTPase), which acts directly on the pool of intracellular nucleotides to prevent misincorporation of uracil residues into DNA by maintaining a low ratio of dUTP to dTTP.

Genomes of DNA viruses like poxviruses and herpesviruses family encode both uracil DNA glycosylase and dUTPase enzymatic activities. Studies analyzing the propagation in cell culture of UDG-deficient pox- or herpesviruses revealed a decrease of infectivity, suggesting an important role for the virally encoded UDG in the viral life cycle (Pyles and Thompson, 1994; Ellison et al., 1996; Prichard et al., 1996; Courcelle et al., 2001). Genomes of Beta retroviruses and nonprimate lentiviruses encode only dUTPase (Elder et al., 1992). Nonprimate lentiviruses efficiently infect nondividing cells, in which the level of dUTP is high, thus enhancing the probability of misincorporating uracil residues during the retrotranscription process. Incorporation into DNA of uracil opposite to guanine generates a promutagenic U:G mismatch, which if not corrected, will lead to a G-to-A transition mutation in the next round of DNA replication (Lindahl, 1993). Although the incorpora-

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tion into DNA of uracil opposite to adenine is not directly mutagenic, uracil in place of thymine in DNA promoter sequences can alter driven gene expression (Verri et al., 1990; Focher et al., 1992). The presence of virally encoded dUTPase indicates that these viruses can locally control the level of dUTP and prevent incorporation of uracil residues into their genome. Studies analyzing the replication of dUTPase-deficient nonprimate CAEV (caprine arthritis-encephalitis virus) lentiviruses showed an accumulation of G-to-A mutations in the viral genome and a loss of pathogenicity (Turelli et al., 1997). dUTPase-deficient nonprimate EIAV (equine infectious anemia virus) lentiviruses exhibited a reduced replication in macrophages (Steagall et al., 1995). Altogether, these data demonstrate that the control of uracil level via the virally encoded UDG or dUTPase activity is crucial for viral replication.

Genomes of primate lentiviruses encode neither dUTPase nor uracil DNA glycosylase, although they too infect nondividing cells. In a previous report, we have demonstrated that host UNG2 is incorporated into HIV-1 viral particles through a specific interaction with IN (Willetts et al., 1999). The presence of host UNG2 into HIV-1 virions highly suggest a role of this DNA repair enzyme in early events of the HIV-1 life cycle. This localization is reminiscent to the presence of virally encoded dUTPase into virions from nonprimate lentiviruses, and we have proposed that UNG2 might have a role similar to that played by the dUTPase in preventing the HIV-1 viral genome toward a genetic drift.

In this study, we wondered whether closely related primate lentiviruses, HIV-2_{ROD} and SIV_{MAC239} have the ability to incorporate host UNG2 into viral particles. We show that both HIV-2 and SIV_{MAC} virions were UNG2-deficient, indicating that HIV-1 have evolved quite differently than HIV-2 and SIV_{MAC} regarding the ability to incorporate UNG2. HIV-1/SIV IN chimeric viruses were impaired for packaging of UNG2 and were impaired for viral propagation.

Results and discussion

In a previous study, we have demonstrated that UNG2 is incorporated into HIV-1 viral particles through a specific interaction with IN (Willetts et al., 1999). We wondered whether IN from related primate lentiviruses shared the ability to bind UNG2. INs from HIV-1_{NL43}, HIV-2_{ROD}, and SIV_{MAC239} molecular clones fused to glutathione *S*-transferase (GST) were bacterially expressed, coupled to glutathione (GSH) agarose beads and GST-IN derivatives were used in GST pull-down assays with recombinant purified UNG2. As shown in Fig. 1A (left), UNG2 bound to GST–HIV-1 IN, and in a lesser extent to GST–HIV-2 IN and GST–SIV IN. The control GST alone was unable to retain UNG2. Similar amounts of GST–IN derivatives were used as judged by Coomassie blue staining (Fig. 1A, right).

To investigate the interaction of IN from HIV-1, HIV-2, and SIV_{MAC} with UNG2 in a cellular context, HeLa cells previously infected with recombinant vaccinia virus expressing T7 polymerase were transfected with plasmids expressing UNG2 and each of the GST–IN fusion proteins cloned under the control of the T7 polymerase promoter. Cell extracts expressed similar level of each of the GST–IN derivatives and similar amounts of UNG2 (Fig. 1B, left and middle). Cell lysate was then incubated with GSH–agarose beads to precipitate GST–IN fusion proteins. Beads were then recovered by centrifugation, and bound proteins were revealed by Western blotting with anti-UNG antibody. UNG2 was present in the precipitated complexes containing GST–HIV-1 IN, while precipitated complexes containing GST–HIV-2 IN or GST–SIV_{MAC} IN displayed lower levels of UNG2 (Fig. 1B, right). As a control, no UNG2 was found associated with unfused GST proteins. These results indicate that UNG2 preferentially associates with IN belonging to HIV-1 strain.

Although our results indicate that UNG2 is preferentially associated with IN from HIV-1 virions, the possibility exists that the weak interaction of UNG2 with HIV-2 and SIV_{MAC} IN might be sufficient to allow the packaging of host UNG2 into these viruses. To demonstrate this possibility, HIV-1, HIV-2, and SIV_{MAC} viruses were purified by sucrose gradient velocity, and viral lysate was resolved on SDS–PAGE and assayed by Western blotting for the presence of packaged UNG2. As a control, the purified recombinant mitochondrial (UNG1) or nuclear (UNG2) isoforms of uracil DNA glycosylase (a gift from G. Slupphaugh) and a whole-cell extract were run in parallel. As shown in Fig. 2 (top), we failed to detect the presence of UNG2 within HIV-2 and SIV_{MAC} virions, while UNG2 was clearly detectable within HIV-1 virions. UNG2 into virions comigrates with the UNG2 but not with the UNG1 isoform, indicating that UNG1 sequestrated into mitochondria was not a substrate for IN. Similar amounts of viruses were analyzed as judged by the presence of capsid (CA) antigen revealed by Coomassie blue staining (Fig. 2, bottom). These results demonstrate that virion-associated UNG2 is specific for HIV-1 strain, but not for HIV-2 or SIV_{MAC} viral strains.

To confirm the role of IN in the packaging of UNG2, we constructed chimeric HIV-1/SIV viruses in which the region encompassing amino acids 6 to 202 of HIV-1 IN was replaced by the one belonging to SIV_{MAC} molecular clone (Fig. 3A). Viral stocks were obtained by transfection, purified by ultracentrifugation, and assayed by Western blotting with anti-UNG antibody. As a control, wild-type HIV-1 (WT) and integrase-deficient HIV-1 (ΔIN) virions were analyzed in parallel. As expected, ΔIN viruses and HIV-1/SIV IN chimeric viruses failed to incorporate UNG2 (Fig. 3B), in contrast to wild-type HIV-1 virions. Moreover, Western blot analysis with anti-Gag and anti-IN antibodies confirmed that the Gag and Gag-Pol precursors are normally processed in chimeric viruses. In addition, the Vpr protein was normally incorporated into HIV-1/SIV IN chimeric

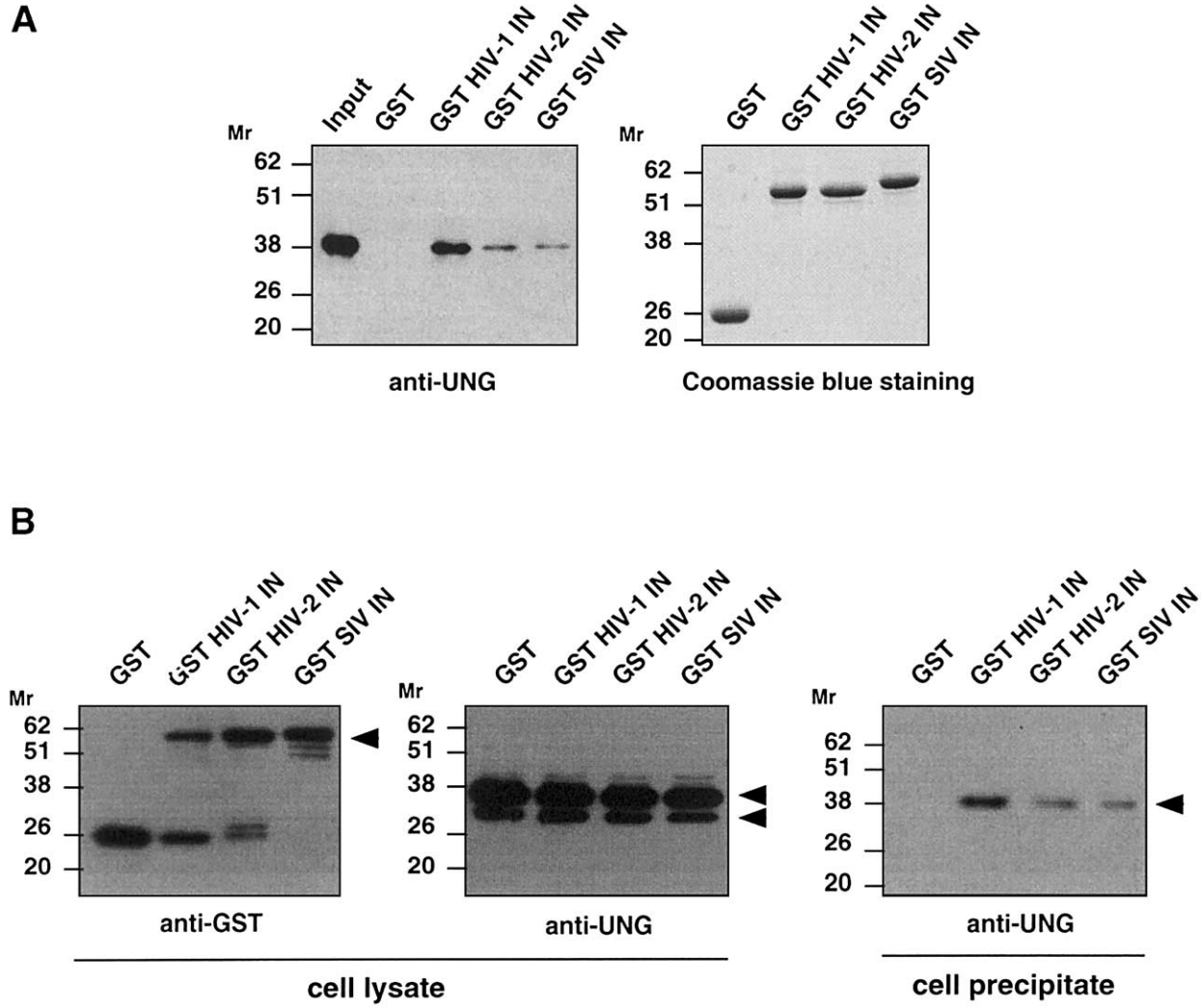

Fig. 1. Association of UNG2 with IN from primate lentiviruses. (A, left) Recombinant purified 6×His-tagged UNG2 was incubated in vitro with equivalent amounts of GST, GST–HIV-1 IN, GST–HIV-2 IN, or GST–SIV_{MAC} IN fusion proteins affinity purified on GSH–agarose beads. After washes, bound proteins were analyzed by Western blot with anti-UNG antibody. (Right) Amounts of GST–IN derivatives were visualized by Coomassie blue staining. Lane marked input contains one-fifth of UNG2 before binding to GST. (B) Recombinant vaccinia virus-infected HeLa cells were cotransfected with expression plasmids containing the T7 polymerase promoter and encoding UNG2 and each of the GST–IN derivatives. The level of expression of GST, GST–IN derivatives, and UNG2 in cell lysate was analyzed by Western blotting with anti-GST and anti-UNG2 antibody (left and middle). GST–IN derivatives were precipitated by addition of GSH–agarose beads, and upon extensive washes, bound proteins were resolved by SDS–PAGE and revealed with anti-UNG antibody (right panel). Mr, molecular mass markers (in kilodaltons).

viruses, confirming that these viruses did not exhibit obvious protein alterations.

In this article, we demonstrate that the cellular UNG2 enzyme, which plays a role in eliminating uracil residues misincorporated into DNA, was packaged into HIV-$1_{NL4.3}$ viral particles, but not into HIV-2_{ROD}, SIV_{MAC239}, or HIV-1/SIV_{MAC} IN chimeric virions, and that IN was the sole viral determinant required for the incorporation of UNG2 into viral particles. Although the UNG2 packaging is a property shared by HIV-1 strains belonging to clade B (NL4.3 and AD8) and clade D (NDK) (this study and Willetts et al., 1999), it is still not known whether this process is a general feature for all HIV-1 clades. The fact that HIV-2 or SIV_{MAC} virions did not incorporate host UNG2 suggests that they might have evolved quite differently from HIV-1. It is possible that UNG2 may associate with INs of HIV-2 and SIV_{MAC} after entry into the cytoplasm of infected cells rather than during budding from the producing cells. Consistent with this, we showed that both HIV-2 and SIV_{MAC} IN still retained the ability to interact with UNG2 in cell as demonstrated by precipitation experiments. Alternatively, HIV-2 and SIV_{MAC} may have the ability to package another class of uracil DNA glycosylases.

Concerning the functional significance of HIV-1-associated UNG2, it is well known that the retrotranscription process is a very early events in the HIV-1 life cycle that takes place in viral particles to promote at least the minus strong-stop cDNA product. It is thus conceivable that the

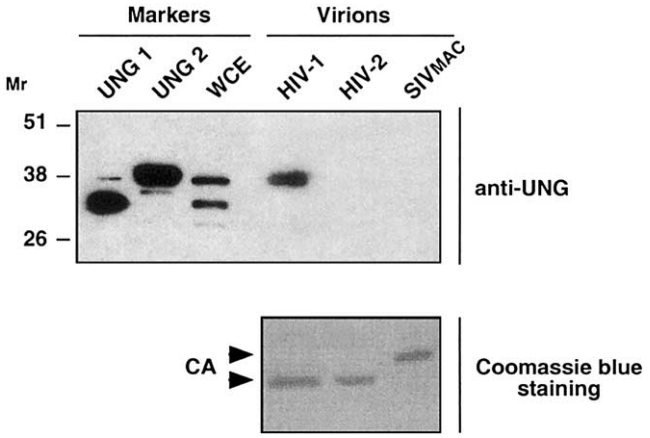

Fig. 2. Incorporation of host UNG2 into HIV-1 but not into HIV-2 and SIV_{MAC} virions. 293T cells were transfected with HIV-1, HIV-2, and SIV_{MAC} molecular clones, and viruses produced in cell-free supernatant were collected by ultracentrifugation and resolved on a 20% to 60% sucrose velocity gradient. Gradient fractions coinciding with the peak of reverse transcriptase activity were pooled, and amounts of viruses were normalized by measuring reverse transcriptase activity. Viruses were solubilized in sample buffer and viral lysate was analyzed by Western blot for the presence of UNG2. Recombinant UNG1 and UNG2 proteins and a whole-cell extract from 293T cells were run in parallel (top). Amounts of viral lysate were estimated by the presence of capsid (CA) antigen revealed by Coomassie blue staining (bottom). Mr, molecular mass markers (in kilodaltons).

presence of this DNA repair enzyme is required at the site of nascent DNA synthesis to control the fidelity of this immediate early retrotranscription. In the case of HIV-2 and SIV, the association of UNG2 and IN in cell might be sufficient to ensure fidelity replication. Studies are in progress to demonstrate that HIV-1-associated UNG2 plays a role similar to that of its cellular counterpart to control the level of misincorporated uracil residues in DNA.

We and others (Bouhamdan et al., 1996; Selig et al., 1997; Sleigh et al., 1998) have reported that UNG2 associates tightly with the HIV-1 Vpr protein. In further studies, we have demonstrated that HIV-1 viral particles engineered to be devoid of virion-associated Vpr still retain the ability to incorporate host UNG2, indicating that Vpr was likely not required for this process (Willetts et al., 1999). In this study, we confirm by using HIV-1/SIV IN chimeric viruses that Vpr did not play an essential role to incorporate endogenously expressed UNG2 into virions, although a cooperation of Vpr with IN in this process could not be excluded. Recent report have indicated that UNG2 expressed in trans in cells could be docked into HIV-1 viral particles via Vpr and participated in the reverse transcription accuracy (Mansky et al., 2000). This unexpected UNG2 packaging via a Vpr-dependent pathway could be a consequence of the abnormally high concentration of both Vpr and UNG2 in the cytoplasm as they are overexpressed by the very efficient pAS1B plasmid (Mansky et al., 2000), since we demonstrated here that endogenously expressed UNG2 was exclusively docked via a IN-dependent pathway. Moreover, Mansky and collaborators did not use UNG-specific antibodies, but HA antibodies, and thus could only looked at HA-tagged-exogenous UNG2, overlooking the endogenous UNG2 still packaged by the IN pathway. Had they used truly UNG2 minus virus in their study, the difference in mutation rate relative to that of their HA–UNG2 plus virus would have been possibly even higher.

The functional role of the Vpr–UNG2 association still remains to be fully understood, however. In addition to its role in the modulation of the mutation rate, the Vpr–UNG2 association could play a role in the nuclear import of the preintegration complex. Two isoforms of UNG differing in their N-terminal extremity, UNG2, which contains a nuclear localisation signal, and UNG1, which contains a mitochondrial localization signal (Otterlei et al., 1998), can associate with Vpr. It has been reported that Vpr, in addition to target nucleus, can target mitochondria and induce apoptosis (Jacotot et al., 2000). The possibility exists that this differential targeting may be under the influence of the differential association of Vpr with UNG2 or UNG1 in infected cells.

A previous study has shown that HIV-1 viruses containing an HIV-2 IN coding sequence exhibited a marked decrease of infectivity (2 orders of magnitude) compared to wild-type HIV-1 (Liu et al., 1999). We observed that HIV-1/SIV chimeric viruses were unable to propagate in culture, indicating that HIV-1 containing an SIV IN coding sequence did not support infectivity (data not shown). At this stage, we do not know whether HIV-1/SIV IN chimeric virus infection was blocked because the chimeric Gag-Pol protein was not correcly folded inducing a defect in late stages of the viral life cycle or whether heterologous SIV IN itself, in the context of HIV-1, was impaired to ensure viral DNA synthesis and integration during the early stages of the viral life cycle. In addition to these possible defects, the absence of virion-associated UNG2 may participate in the loss of infectivity.

Materials and methods

Plasmids

Viral molecular clones used in this study were those from $HIV\text{-}1_{NL43}$, $HIV\text{-}1_{AD8}$, $HIV\text{-}2_{ROD}$, and SIV_{MAC239}. To generate the chimeric HIV-1 molecular clone with the IN sequence from SIV, the 3743-bp *Apa*I–*Eco*RI of $pHIV\text{-}1_{AD8}$ was subcloned in pBluescript. An artificial unique *Cla*I restriction site was then introduced to modify the 15th nucleotide (A versus C) of the IN open reading frame without affecting the codon. The 708-bp *Cla*I–*Hin*dIII fragment of HIV-1 IN was deleted by enzymatic digestion and replaced by the SIV_{MAC} counterpart amplified with primers containing *Cla*I and *Hin*dIII restriction sites, respectively (5′-ATACCATCGATCCAGCACAAGAAGAACATGATAAA-3′ and 5′-AGCCCCAAGCTTACCGGGTCCCTTCCACAGTTG-3′). The 120-bp *Pac*I–*Hin*dIII fragment from

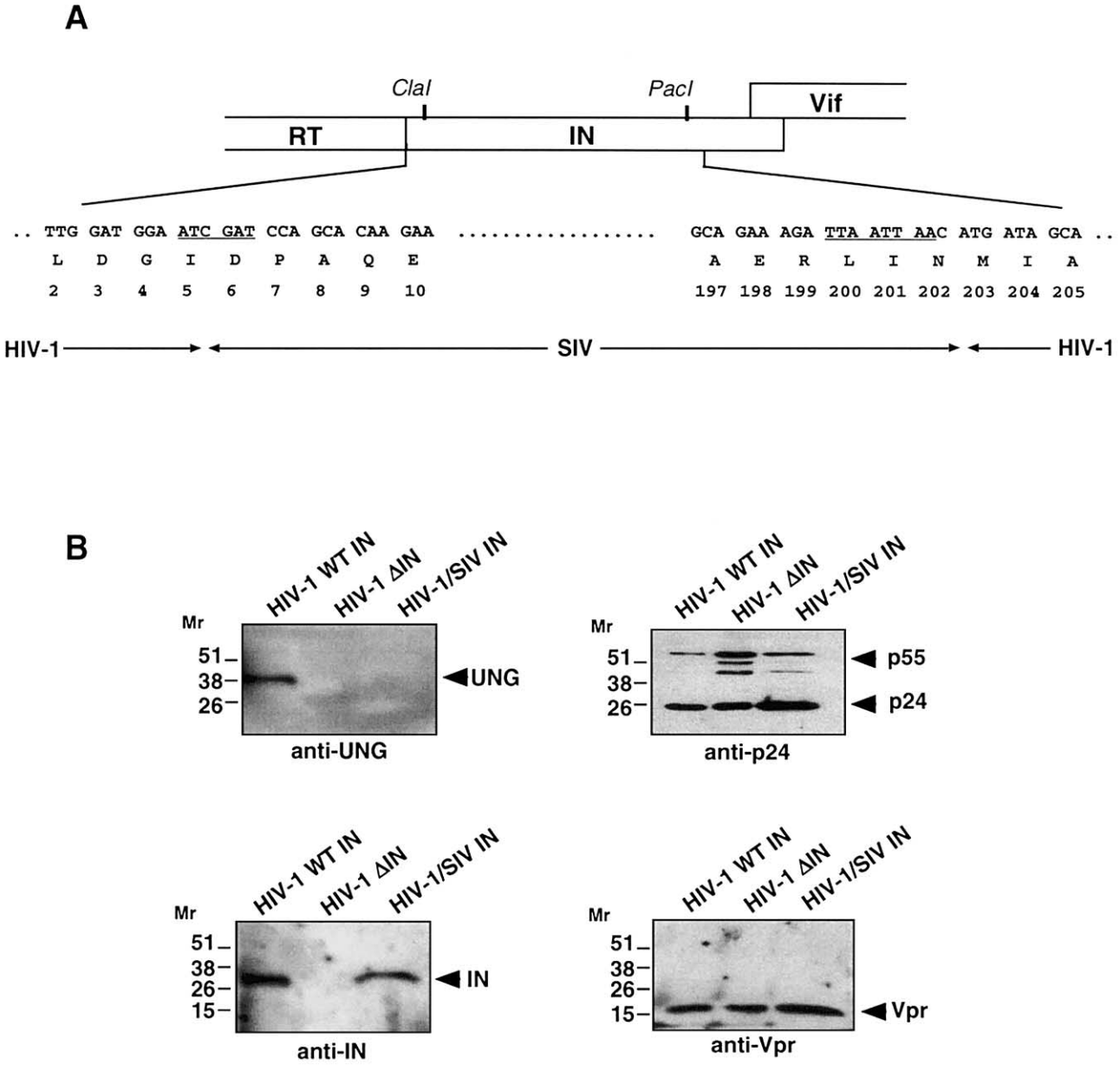

Fig. 3. HIV-1/SIV_{MAC} viruses containing chimeric IN were deficient for UNG2 packaging. (A) Schematic diagram showing the chimeric HIV-1 IN containing amino acids 6 to 202 from SIV_{MAC} N protein. (B) 293T cells were transfected with either wild-type (WT) or integrase-deficient HIV-1 (ΔIN) or chimeric HIV-1/SIV molecular clone containing IN of SIV_{MAC} in the context of HIV-1 background. Viral stocks produced from transfected cells were highly purified on sucrose gradient velocity and analyzed by Western blot for the presence of virion-associated UNG2 and for the expression of Gag, Vpr, and IN proteins. Mr, molecular mass markers (in kilodaltons).

SIV_{MAC} IN was then deleted by enzymatic digestion and replaced by the same fragment from HIV-1 IN obtained by PCR amplification with a primer containing *Pac*I restriction site (5′-GGG CAG AAA GAT TAA TTA ACA TGA TAG CAA CAG ACC TAC AAA CTA AA-3′) and the primer containing *Hin*dIII restriction site. Finally, the *Apa*I–*Eco*RI fragment was cloned back into pNL43 plasmid to obtain a HIV-1 molecular clone encoding a chimeric HIV-1 IN gene with residues 6 to 202 belonging to the SIV_{MAC} strain. The chimeric HIV-1/SIV IN was checked by sequencing. To obtain the HIV-1 ΔIN construct, the *Cla*I restriction site located into HIV-1 IN was filled in, and the resulting construct encodes only the five first residues of IN. Plasmids allowing the expression in human cells of GST–IN derivatives and UNG2 under the control of the T7 polymerase promoter and plasmids allowing prokaryotic expression of GST–IN derivatives were as previously described (Willetts et al., 1999).

Cell culture and transfection

Human 293T and HeLa cells were cultured in DMEM (Gibco) supplemented with antibiotics and 10% FCS. Subconfluent 293T cells were transfected by the FuGENE 6 transfectant reagent (Roche) according to the manufacturer's protocol. For coprecipitation studies, subconfluent HeLa cells were infected for 30 min with 1 PFU of recombinant vaccinia virus (T7-MVA) (a gift from G. Sutter) per cell to express T7 RNA polymerase and then transfected with plasmids expressing GST–IN derivatives and UNG2 cloned under the control of the T7 polymerase promoter.

Protein–protein interactions

The recombinant 6×HIS–UNG2 protein was expressed in bacteria and affinity-purified on nickel column according to the manufacturer's recommendations. For GST pull-down assays, 5 μg of immobilized GST–IN derivatives was incubated with 100 ng of purified 6×HIS–UNG2 in binding buffer as previously described (Willetts et al., 1999). Bound proteins were resolved on SDS–PAGE and revealed by Western blotting with rabbit polyclonal anti-UNG antibody (a gift of G. Slupphaug). For in vivo coprecipitation experiments, extracts from HeLa cells infected with vaccinia recombinant plasmids and transfected with mammalian expression plasmids encoding GST–IN derivatives and UNG2 were harvested 20 h posttransfection and lyzed by three freeze–thawing cycles in a buffer containing 20 mM Tris–HCl, pH 7.8, 100 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.5% NP-40, and a cocktail of antiprotease (Roche). An aliquot of cell lysate was used to visualize expression of GST–IN derivatives with rabbit polyclonal anti-GST (Santa Cruz Biotechnologies). GSH–agarose beads were then added to the cell lysate and then extensively washed. Bound proteins were revealed by Western blotting with anti-UNG antibody.

Virion purification and Western blotting

Viral stocks were obtained by transfection of 293T cells with plasmids expressing each of the viral molecular clones. Forty-eight hours posttransfection virions produced in the cell-free supernatant were collected by ultracentrifugation and purified through a 20%–60% sucrose gradient velocity. Gradient fractions coinciding with the peak of reverse transcriptase activity were pooled and normalized for equivalent amounts of reverse transcriptase activity. Virus pellet was solubilized in sample buffer, and viral lysate was resolved on 12% SDS–PAGE and analyzed by Western blot with rabbit polyclonal anti-UNG, rabbit polyclonal anti-IN (a gift from D. Trono), and rabbit polyclonal anti-Vpr (a gift of N. Landau). The primary antibody was revealed by horseradish peroxidase (HRP)-conjugated secondary antibody (Dako) and HRP activity was detected by enhanced chemiluminescence (ECL+, Amersham).

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